INHIBITORS OF APOPTOSIS AFFECT DNA DEGRADATION AND REPAIR IN SULFUR MUSTARD (HD)-EXPOSED HUMAN EPIDERMAL KERATINOCYTES (HEK)

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ABSTRACT

In cultured HEK, we observed that HD (0.3-1.0 mM)-induced DNA damage triggers DNA repair and apoptosis, which may be interdependent. We studied the effects of a general caspase inhibitor, Z-VAD-fmk (0.004 mM) and the Fas (CD95) receptor (induces apoptosis on Fas ligand binding) antibody on DNA damage and its repair in HD-exposed HEK. Both Z-VAD-fmk and the CD95 antibody reduced HD-induced DNA degradation possibly by decreasing DNA degradation or enhancing DNA repair or both. These inhibitors may be useful for modulating DNA repair and apoptosis in HD-exposed cells with potential applications in medical management of HD-induced vesication.

INTRODUCTION

Sulfur mustard (HD) is a vesicant that introduces single- and double-strand breaks in cellular DNA along with the formation of alkyl adducts. Our previous work has revealed that the HD-induced DNA damage is repaired fairly rapidly within about 5 hr. This repair is accompanied by DNA ligase I activation via DNA-dependent protein kinase (DNA-PK) mediated phosphorylation, and is retarded in the presence of a poly (ADP-ribose) polymerase (PARP) inhibitor or a calcium chelator (Bhat *et al*., 1998, Bhat *et al*., 1999, Bhat *et al*., Manuscript in preparation). The cells following damage to their DNA can activate either the DNA repair pathway or the apoptotic pathway. Activation of the repair pathway may lead to the recovery of cells from the damage and normal proliferation. However, this does not guarantee total fidelity of repair and the probability of cellular transformation is high (Kuo *et al*., 1999).

The primary event in the initiation of apoptosis is the proteolytic cleavage of PARP by caspase-3 (Rosenthal *et al*., 1998). DNA ladder formation has been used as a marker for apoptosis (Krammer, 2000). The cell surface glycoprotein CD95 is a member of the death receptor family of proteins with a role in the immune system. CD95-induced apoptosis is initiated by its natural ligand CD95L, and oligomerization of CD95 is required for apoptotic signal transduction (Krammer, 2000). PARP is essential for DNA repair and lack of PARP retards DNA repair (Bhat *et al*., 2000). The apoptotic signaling that results in caspase-3 activation can be blocked by CD95 antibody. A cell permeable tripeptide z-VAD-fmk (benzyl oxycarbonyl-valinyl-alanyl-aspartyl (O-methyl-fluromethylketone)) also

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has an apoptosis inhibiting effect (An & Knox, 1996). In the presence of these inhibitors, the cell following HD damage may repair rapidly and continue on the normal cell cycle. The results of these studies may be useful for developing strategies for a medical intervention of HD damage.

EXPERIMENTAL METHODS

Chemicals and Cells: HD (> 98% pure) was obtained from the US Army Soldier Biological and Chemical Command, Aberdeen Proving Ground, MD. HEK and keratinocyte growth medium (KGM) were from Clonetics Corp., San Diego, CA and the cell lines were obtained from American Type Culture Collection. $^{33}PO_4$ (specific activity, 8810 Ci/mmol, 1mCi/ml) was obtained from NEN, Boston, MA. Oligo dT cellulose was obtained from Sigma Chemicals, St. Louis, MO. Terminal transferase was purchased from Promega, Madison, WI. ^{3}H oligo dT was synthesized according to the procedure described previously (Bhat *et al*., 1998). All other chemicals were of the purest grade available. CD95 antibody secreting hybridoma M3 was obtained from American Type Culture Collecction (ATCC No. HB 11726). Bovine DNA ligase I monoclonal antibody was a kind gift from Dr. Tomas Lindahl of the Imperial Cancer Research Fund, UK.

Cell Culture: Frozen stock HEK (passage 2, 5 x 10^5 cells/vial) were cultured in 150 cm^2 tissue culture flasks (10^5 to 3 x 10^5 cells/flask) to initiate the culture. Confluent monolayer cells were used in the experiments. HEK from a single donor subcultured to passage 3 only was used. Hybridoma were cultured in DMEM + 10% fetal calf serum containing 100 units of penicillin and 100 µg of streptomycin per ml.

Exposure of HEK to HD and metabolic ^{33}P labeling of DNA ligase in HEK and other cells: The experimental and control cells were washed with 37^{o}C saline and then exposed to 1 mM HD after incubation at 37^{o}C for 30 min with z-VAD-fmk in a phosphate-free medium (148 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 10 mM glucose, 25 mM HEPES, pH 7.4, 326 mOHDol/Lt) or for one hr with CD95 antibody. HD stock (4 mM) obtained as a frozen globule in saline was thawed, dissolved and then applied to the washed monolayer of HEK along with $^{33}PO_4^{-3}$. For preparation of cell-free extracts (CFE), the cells were exposed in a 37^{o}C phosphate-free medium containing 0.5 mCi of $^{33}PO_4^{-3}$ in a total volume of 10 ml/150 cm^2 flask using HD obtained in saline and then set aside for 30 min at room temperature followed by incubation for two hr at 37^{o}C in a CO_2 incubator. Following this, the medium and the label were removed, the cells washed with ice cold saline and then collected by scraping of the flasks. The cells were homogenized in an extraction buffer containing the following: 300 mM NaCl, 50 mM Tris.HCl, pH 7.5, 1 mM each of EDTA and DTT, 0.1% Triton X-100 and 10% glycerol. Following protease inhibitors at the stated concentration were also present: phenyl methyl sulfonyl fluoride, 1 mM; pepstatin, 5 µg/ml; aprotinin, 2 µg/ml; leupeptin, 1.5µg/ml and N-alpha-P-tosyl-L-lysine chloromethyl ketone, 0.5 µg/ml (Bhat *et al*., 1998). The cells were harvested after 2 hrs of incubation at 37^{o}C in a CO_2 incubator and suspended in the extraction buffer and homogenized by vortexing and several freeze-thaw cycles. The CFE was then assayed for DNA ligase activation by affinity chromatography of DNA ligase I to determine the labeling pattern of DNA ligase I. Bovine DNA ligase I monoclonal antibody was used to prepare the affinity column.

DNA isolation and analysis: The cellular DNA was isolated from the HD-exposed and HD-unexposed HEK using a standard DNA extraction kit and then analyzed on a 0.8% alkaline agarose gel and the images were recorded digitally using a Kodak image analyzer.

Purification of CD95 antibody: CD95 hybridoma culture medium was used as the source. Proteins precipitating at 40% ammonium sulfate from the culture medium were used to purify the antibody using a protein A column obtained from Biorad laboratories.

DNA ligase and protein assays: DNA ligase was assayed using poly dA-^{3}H oligo dT substrate as previously described (Bhat *et al*., 1998). Protein was assayed using the Biorad protein assay reagent and bovine γ-globulin was the standard.

RESULTS AND DISCUSSION

We examined the effect of the general caspase inhibitor z-VAD-fmk on DNA degradation using agarose gel analysis, and in a more sensitive fashion using DNA ligase I activation by phosphorylation. We have previously demonstrated that DNA ligase I is phosphorylated and activated by DNA-PK in HD-exposed HEK and this activation is specific to DNA double-strand breaks introduced by HD. The apoptotic events cause DNA degradation. DNA repair is inhibited by PARP degradation by caspase-3 and PARP is essential for the repair to proceed. Therefore, apoptosis inhibitors that prevent PARP degradation are expected to inhibit DNA degradation and enhance DNA repair.

In Fig. 1, the ^{33}P labeling profiles (elution of ^{33}P-labeled protein from DNA ligase I monoclonal antibody affinity column) of DNA ligase I in HD-unexposed HEK, HD-exposed HEK, and HD-exposed HEK in the presence of z-VAD-fmk are shown. Increased ^{33}P labeled DNA ligase I indicates DNA double-strand breaks. It is clear that in the presence of z-VAD-fmk, DNA ligase I activation is reduced indicating a decrease in DNA double-strand breaks.

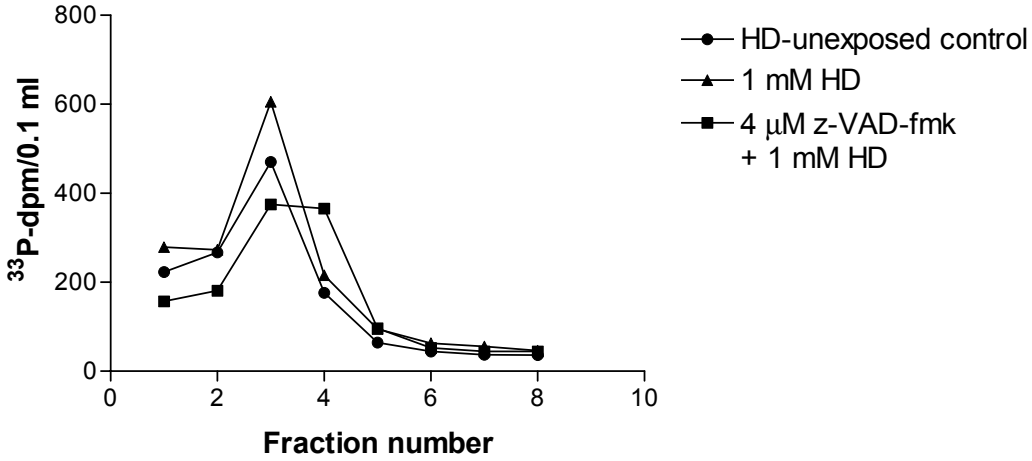

Figure 1.The general caspase inhibitor z-VAD-fmk decreases DNA degradation due to HD

We examined the DNA isolated from the same cells by alkaline agarose gel electrophoresis and lane analysis of the bands utilizing the Kodak image station 440 and the Kodak 1D gel analysis software. We found using normal analysis sensitivity that high molecular weight bands accumulated in the DNA samples from cells exposed to HD in the presence of z-VAD-fmk compared to a single lower molecular weight band (smear) in DNA from cells exposed to HD alone. Increasing the sensitivity revealed additional bands in the z-VAD-fmk sample, but still a single band (smear) in the HD alone sample. One possible explanation is that z-VAD-fmk inhibits caspase-3 and, hence, PARP degradation, and therefore facilitates DNA repair. This supports the observation made with the more sensitive DNA ligase activation assay described and the results shown in figure 1. These results show that by inhibiting apoptosis, DNA repair can be enhanced and the survival of HD-exposed cells can be promoted.

The results (elution of ^{33}P-labeled protein from DNA ligase I monoclonal antibody affinity column) shown in Fig. 2 indicate that in the presence of CD95 antibody HD-induced DNA ligase I phosphorylation is inhibited. This implies that (a) DNA-PK activation that is dependent on DNA double-strand break did not occur, and/or (b) the CD95 signaling pathway may somehow be involved in HD-induced DNA degradation.

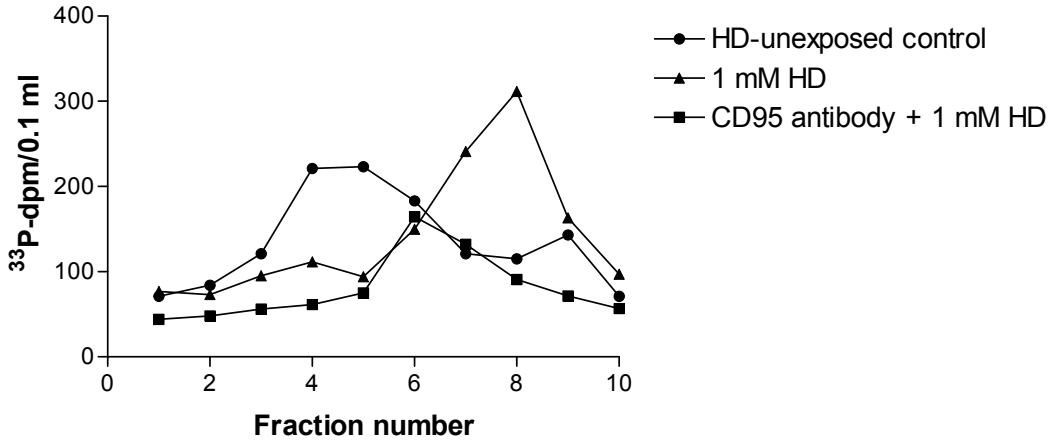

Figure 2. The CD95 antibody prevents DNA degradation due to HD

CONCLUSIONS

In this study, we tested the effects of two types of apoptosis inhibitors on DNA degradation and repair in HD-exposed HEK. The general caspase inhibitor z-VAD-fmk decreased HD-induced DNA degradation (double-strand breaks), and also promoted DNA repair. Pretreatment of HEK with antibodies to the cell surface glycoprotein CD95 prevented HD-induced DNA degradation. This observation suggests that CD95 is involved in the signaling of the initiation of DNA degradation. The ability of apoptosis inhibitors to modulate DNA damage and repair in HD-exposed HEK may be utilized to develop antiapoptotic pharmacological interventions for protection against vesicant injury.

REFERENCES

1. An, S. and Knox, K. A., Ligation of CD40 rescues Ramos-Burkitt lymphoma B cells from calcium ionophore- and antigen receptor-triggered apoptosis by inhibiting activation of the cysteine protease CPP32/Yama and cleavage of its substrate PARP. FEBS Lett., **386**: 115-122, 1996.

2. Bhat, K. R., Benton, B. J. and Ray, R., DNA ligase activation following sulfur mustard exposure in cultured human epidermal keratinocytes. In Vitro & Mol. Toxicol., **11**: 45-53, 1998.

3. Bhat, K. R., Benton, B. J. and Ray, R., DNA ligase I activation and repair of alkylation damaged DNA is via DNA-dependent protein kinse (DNA-PK) mediated phosphorylation. FASEB J., **13**: A 696, 1999.
4. Bhat, K. R., Benton, B. J., Rosenthal, D. S., Smulson, M. and Ray, R., The role of poly (ADP-ribose) polymerase (PARP) in DNA repair in sulfur mustard-exposed normal human epidermal keratinocytes. J. Appl. Toxicol., **20**: S13-S17, 2000.

5. Bhat, K. R., Benton, B. J. and Ray, R., DNA ligase I is a natural substrate for DNA-dependent protein kinase and is activated by phosphorylation in response to DNA double-strand breaks (manuscript in preparation).

6. Krammer, P. H., CD95's deadly mission in the immune system. Nature, **407**: 789-795,
7. 2000.

8. Kuo, M-L, Shiah, S-G, Wang, C-J and Chuang, S-EN, Suppression of apoptosis by Bcl-2 to enhance benzene metabolite–induced oxidative DNA damage and mutagenesis: A possible mechanism of carcinogenesis. Mol. Pharmacol., **55**: 894-901, 1999.

9. Rosenthal, D. S., Simbulan-Rosenthal, C. M. G., Iyer, S., Spoonde, A., Smith, W., Ray, R. and Smulson, M. E., Sulfur mustard induces markers of terminal differentiation and apoptosis in keratinocytes via a Ca^{2+}-calmodulin and caspase-dependent pathway. J. Invest. Dermatol., **111(1)**: 64-71, 1998.